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# Endosomal toll-like receptors play a key role in activation of primary human monocytes by cowpea mosaic virus

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## Summary

**The plant virus, cowpea mosaic virus (CPMV), has demonstrated a remarkable capacity to induce anti-tumour immune responses following direct administration into solid tumours. The molecular pathways that account for these effects and the capacity of CPMV to activate human cells are not well defined. Here, we examine the ability of CPMV particles to activate human monocytes, dendritic cells (DCs) and macrophages. Monocytes in peripheral blood mononuclear cell cultures and purified CD14+ monocytes were readily activated by CPMV *in vitro*, leading to induction of HLA-DR, CD86, PD-L1, IL-15R and CXCL10 expression. Monocytes released chemokines, CXCL10, MIP-1α and MIP-1β into cell culture supernatants after incubation with CPMV. DC subsets (pDC and mDC) and monocyte-derived macrophages also demonstrated evidence of activation after incubation with CPMV. Inhibitors of spleen tyrosine kinase (SYK), endocytosis or endocytic acidification impaired the capacity of CPMV to activate monocytes. Furthermore, CPMV activation of monocytes was partially blocked by a TLR7/8 antagonist. These data demonstrate that CPMV activates human monocytes in a manner dependent on SYK signalling, endosomal acidification and with an important contribution from TLR7/8 recognition.**

**Keywords:** cowpea mosaic virus; endosome inhibitor; monocytes; Syk signalling; TLR7/8.

## Introduction

Plant virus-like nanoparticles (VLPs) provide a potential tool for drug delivery, imaging and immune-based therapies.[1–4] Plant viruses can be produced at low cost, are relatively easy to engineer, are pH and thermally stable, and are non-infectious to human tissues. Some types of plant viruses that have adjuvant properties in animals are currently being assessed for their potential as a novel type of tumour immunotherapy. The virions and VLPs of cowpea mosaic virus (CPMV) have profound anti-tumour effects when injected into tumours. These effects have been demonstrated in various animal models, and have been associated with induction of host immune mechanisms.[5,6] Similarly, VLPs from another plant virus, papaya mosaic virus (PapMV), significantly slowed

**Abbreviations**: CPMV, cowpea mosaic virus; DC, dendritic cell; eCPMV, empty CPMV particles; LPS, lipopolysaccharide; PapMV, papaya mosaic virus; PBMC, peripheral blood mononuclear cell; SYK, spleen tyrosine kinase; VLPs, plant virus nanoparticles

 

tumour progression and prolonged survival in mice challenged with melanoma tumours.[8] The effects of PapMV were dependent on interferon (IFN)-α.

The structural characteristic of CPMV includes an icosahedral capsid (measuring ~30 nm in diameter) composed of 60 small and large coat protein units each forming a pT = 3 capsid. The particles encapsidate a bipartite, single-stranded RNA genome.[9] Native CPMV can be produced either by mechanical inoculation of their host plant, black-eyed peas, or by agroinfiltration using Australian tobacco plants. The latter method is especially useful for generating genome-free empty CPMV (eCPMV) particles.[10]

Although CPMV has profound effects on tumour immunity in animals, the activity of these particles in primary human cells is unknown. CPMV interacts with human cells. For example, similar to murine macrophages, human macrophages readily internalize CPMV.[2,11,12] Furthermore, CPMV particles that are mixed with human peripheral blood mononuclear cells (PBMCs) preferentially adhere to monocytes and dendritic cells (DCs).[2] Nonetheless, it is not known if CPMV can mediate activation of these cells. Here, we provide evidence that CPMV readily mediates activation of human monocytes, macrophages and DCs. Our findings indicate that the effects of CPMV on cellular activation are dependent on spleen tyrosine kinase (SYK) signalling and toll-like receptor (TLR) activation in the endocytic compartment.

## Materials and methods

### *CPMV propagation and purification*

Cowpea mosaic virus particles were propagated and purified from black-eyed peas No. 5; the methods were as previously described.[13] The particles were characterized by UV/visible spectroscopy, agarose gel electrophoresis, sodium dodecyl sulphate−polyacrylamide gel electrophoresis, and size exclusion chromatography and transmission electron microscopy (Fig. S1). Endotoxin levels were determined after each CPMV preparation using LAL chromogenic endotoxin kit, and only samples with endotoxin levels $< X$ were used in the experiments [$X$ = 0·5 pg/ml lipopolysaccharide (LPS)].

### *Cells*

This work, including blood draw procedures and written consent forms for obtaining whole blood samples from healthy adult volunteers, was approved by University Hospitals of Cleveland Internal Review Board. PBMCs were isolated from whole blood by centrifugation over a Ficoll-Hypaque gradient as previously described.[14] Cells were counted by Beckman Coulter Vi-CELL™ XR Cell Viability Analyzer (Beckman Coulter, Fullerton, CA). RosetteSep and EasySep™ human monocyte isolation kits (StemCell Technologies, Vancouver, BC, Canada) were used according to the kit instructions to isolate human monocytes by negative selection. Purity of isolated monocytes (mean: 90·4%; range: 79·3%–96·9%) was assessed by flow cytometry, measuring percentages of CD14+ cells using a BD LSRFortessa flow cytometer (BD Biosciences, San Jose, CA). PBMCs that were depleted of CD14+ cells with human anti-CD14 microbeads (auto MACS pro cell sorter; Miltenyi Biotec, Auburn, CA), were used for studies of DC responses to CPMV. Monocyte-derived macrophages were obtained by pre-incubating purified monocytes in Roswell Park Memorial Institute 1640 (RPMI 1640) containing 1% penicillin streptomycin, 1% HEPES (Lonza, Basel, Switzerland), 1% L-glutamine and 10% fetal bovine serum. These cell cultures were supplemented with 10 ng/ml rhGM-CSF (Gibco Life Technologies, Carlsbad, CA) and cells were incubated 6 days prior to being used in assays. At this time, recovered cells have marked increase in cell size and granularity as determined by flow cytometry and consistent with macrophage morphology (not shown).

### *Cell stimulation*

To assess cellular activation, PBMCs, purified monocytes, monocyte-derived macrophages or CD14-depleted PBMCs were re-suspended in complete medium and incubated overnight at 37° in the presence or absence of CPMV particles (6 μg/ml), Imiquimod (1 μg/ml; InvivoGen, San Diego, CA) or LPS (50 ng/ml; Sigma Chemical, St Louis, MO). For studies with chemical inhibitors, 1·5 million cells/ml were pre-incubated for 2 hr +/− Syk inhibitor (2 μM/ml; 2,3-dihydro-3-[(1-methyl-1H-indol-3-yl) methylene]-2-oxo-1H-indole-5-sulphonamide; Cayman Chemical, Ann Arbor, MI) or +/− dynasore (50 μM/ml; Abcam, Cambridge, MA). Chloroquine diphosphate (10 μM: Sigma Chemical) and synthetic oligodeoxyribonucleotide, ODN 2087 (TLR7/8 inhibitor, 8 μM; Miltenyi Biotec) were pre-incubated with cells for 1 hr prior to stimulation with CPMV.[15] Following the incubation period, cells were harvested from wells, washed and stained for flow cytometric analysis. Cells were incubated with antibodies for 15 min on ice, and then washed, fixed and permeabilized with 1 × perm/wash buffer (BD Bioscience). Cells were incubated with anti-CXCL10 (FITC); clone#B-C50 (Abcam) for intracellular staining. Cells were then washed with 1 × perm/wash buffer and assessed by flow cytometry with a BD Fortessa instrument.

### *Statistical analysis*

Statistical analyses were performed using Prism (GraphPad Software, San Diego, CA). Wilcoxon matched-pairs signed-


 

ranks test or Mann–Whitney *U*-test were used to determine significant ($P < 0{\cdot}05$) differences in cells pretreated or not with inhibitor, and for comparisons between cells treated with various stimuli, respectively. Kruskal–Wallis tests were used for multi-group comparisons.

## Results

### CPMV activates human monocytes and DCs

To investigate the potential of CPMV to activate human immune cells, we incubated PBMCs overnight with CPMV and assessed the expression of activation markers on CD14+ monocytes using flow cytometric analyses. Changes in cell surface markers were measured as increases in the percentage of positive cells or as increases in MFI following cellular activation. MFI was preferred for markers constitutively expressed in most cells at baseline. CPMV particles were used at 6 μg/ml for most experiments. Dose–response studies indicated that 6 μg/ml was near optimal, with detectable levels of activity observed as low as 100 ng/ml (not shown). Cells were incubated with LPS (TLR4 agonist) or Imiquimod (TLR7 agonist) as positive controls. Stimulation of PBMCs with CPMV resulted in marked increases in the expression of cell surface activation markers, CD86, PD-L1, HLA-DR and IL-15R, in CD14+ cells. As anticipated, monocytes readily responded to stimulation with the positive controls, LPS or Imiquimod (Fig. 1).

Similar to the stimulation of monocytes in PBMCs, we also observed activation of purified monocytes after incubation with CPMV. In both experiments, we included analyses of intracellular CXCL10/IP-10 expression, a chemokine induced by IRF-dependent signalling.[16–19] Purified monocytes readily responded to CPMV stimulation by increasing cell surface expression of CD86, PD-L1, HLA-DR and IL-15R, and by markedly enhancing intracellular expression of CXCL10 (Fig. 2). These data suggest that CPMV can directly mediate activation of human monocytes.

To assess the potential for CPMV to activate other cell professional antigen-presenting cells (APCs), we evaluated activation marker expression in DC subsets from CD14-depleted PBMCs that had been treated with CPMV overnight. DC subsets included CD11c+ CD123low myeloid DCs (mDC) and CD11clowCD123+ plasmacytoid DCs (pDC). CPMV induced expression of CD86 and PD-L1 in both mDC and pDC, although the effects were more pronounced in mDC (Fig. 2a,b). In addition to DCs, we assessed the potential for CPMV to activate monocyte-derived macrophages. These cells were stimulated overnight with CPMV and evaluated for expression of CD86 and PD-L1 as indicators of cellular activation. CPMV readily caused induction of CD86 and PD-L1 in these cells (Fig. S2c). The capacity of CPMV to cause the induction of cytokine and chemokine secretion was assessed in purified monocytes. Monocytes incubated overnight with CPMV released significantly higher levels of CXCL10, MIP-1α and MIP-1β into cell culture supernatants compared with cells incubated in medium alone (Fig. 3a–c). Unlike positive controls (Imiquimod or LPS), CPMV did not significantly enhance release of MCP-1, Gro-α, IL-6, IL-8, TNFα or SDF-1a into cell culture supernatants (Fig. 3d–i). In addition, CPMV did not induce expression of IL-1b, suggesting an absence of inflammasome activation (Fig. 3j).

### Syk signalling is critical for monocyte activation induced by CPMV

Syk is an important signalling molecule involved in various innate receptor signalling pathways, including TLRs such as TLR7 and TLR9.[20] To determine if Syk may play a role in monocyte activation by CPMV, PBMCs were pre-incubated with Syk inhibitor and then stimulated with CPMV overnight. Imiquimod or LPS stimulation was tested for comparison. The addition of Syk inhibitor to CPMV-stimulated PBMCs resulted in inhibition of HLA-DR, CD86, PD-L1, IL-15R and CXCL10 (Fig. 4). Similar results were observed with Imiquimod-stimulated cells, although the effects of the inhibitor were less pronounced. Syk inhibitor tended to only modestly diminish responses to LPS stimulation. Curiously, in contrast to its effects on other activation markers, Syk inhibitor actually caused an enhancement in the induction of CD86 expression in monocytes treated with LPS (Fig. 4).

### Activation of monocytes by CPMV depends at least partly on dynamin activity, endosomal acidification and TLR7/8/9

To investigate the mechanism of CPMV-mediated monocyte activation in more detail, we tested whether activation of primary human monocytes could be blocked by inhibition of endocytosis (dynasore), endosomal acidification (Chloroquine) or by an antagonist of TLR7/8 (ODN 2087). To interfere with endocytosis, PBMCs were incubated with or without dynasore for 1 hr followed by stimulation with or without CPMV, Imiquimod or LPS. The addition of dynasore to cell cultures partially abolished CPMV induction of CD86, PD-L1 and HLA-DR (Fig. S3a–c), but had limited effects on either Imiquimod- or LPS-mediated activation of monocytes (not shown). These data are consistent with a role for endocytosis in the activity of CPMV.

To test for the importance of endosomal acidification on CPMV responsiveness, PBMCs were pretreated with Chloroquine for 1 hr followed by stimulation with or without CPMV, Imiquimod or LPS. The addition of Chloroquine to cell cultures markedly inhibited CPMV or

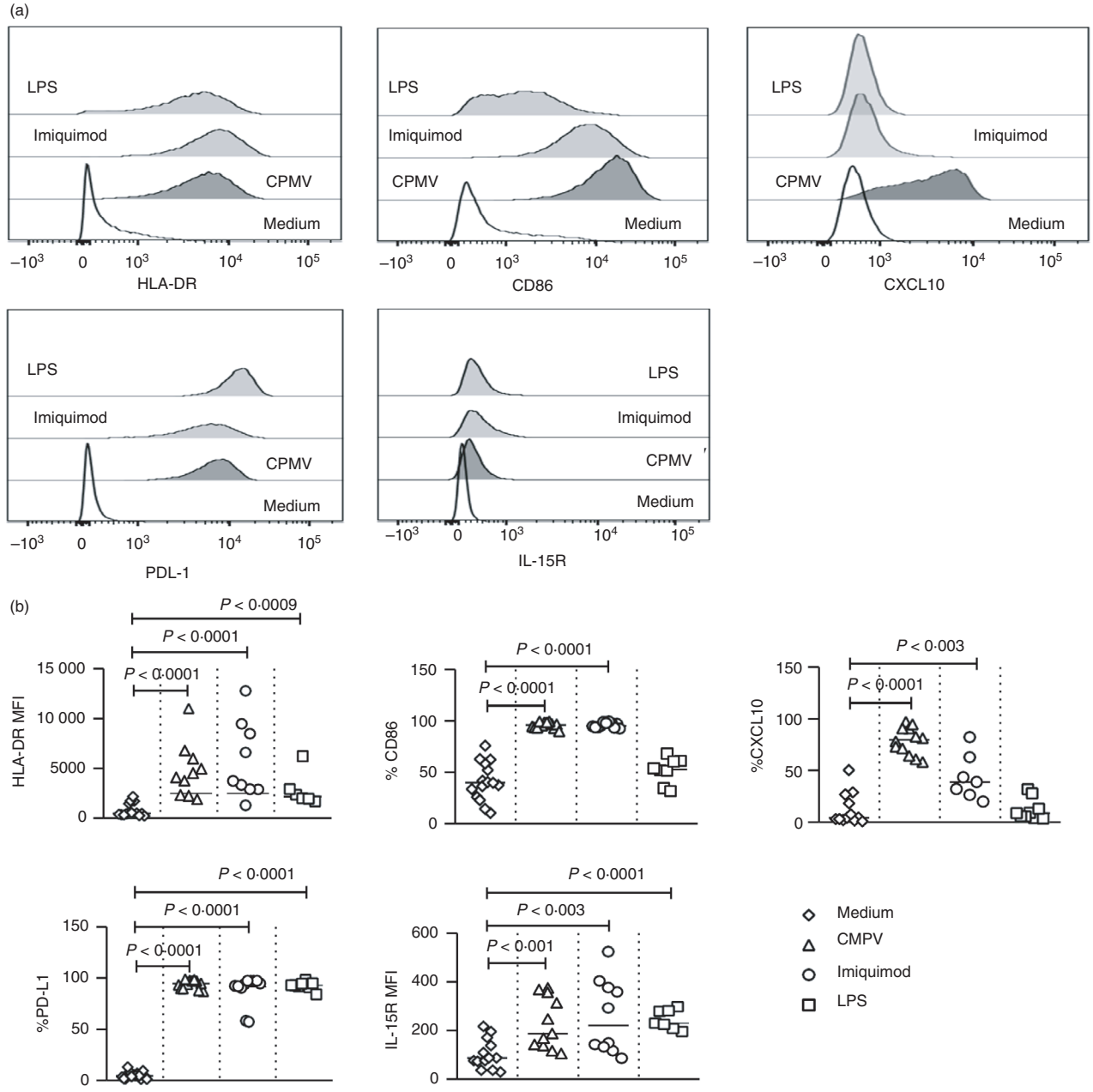


**Figure 1.** Monocytes are activated by cowpea mosaic virus (CPMV). Peripheral blood mononuclear cells (PBMCs) were incubated overnight in complete medium alone or medium supplemented with CPMV (6 μg/ml), Imiquimod (1 μg/ml) or lipopolysaccharide (LPS; 50 ng/ml). (a) For flow cytometric analyses, debris, dead cells and doublets were excluded with FSC versus SSC, FSC-A versus FSC-H and viability dye gating. Histograms are shown for CD14 + monocytes that were stained for expression of CD86, PD-L1, HLA-DR, IL-15R or CXCL10 (*x*-axis). (b) Summary data using cells from different donors showing the medium for monocytes in PBMCs. Samples were evaluated using Mann–Whitney *U*-test. *P*-value < 0·001 by Kruskal–Wallis test.

Imiquimod induction of CD86, IL-15R, HLA-DR and PD-L1 cell surface expression as well as intracellular expression of CXCL10 in CD14+ monocytes; however, Chloroquine had no effect on monocyte activation induced by LPS (Fig. 5a,b).

To assess a specific role for TLR7/8 in monocyte activation by CPMV, we pre-incubated cells with a specific TLR7/8 antagonist, ODN 2087, prior to stimulation with CPMV or other TLR agonists (Imiquimod or LPS). The ODN TLR7/8 inhibitor partially inhibited activation of monocytes by CPMV or Imiquimod, while having little effect on activation induced by LPS (Fig. 5a,c). Together, these data are consistent with a key role for endosomal TLR7/8 in CPMV-mediated activation of human monocytes (Fig. 6).


 

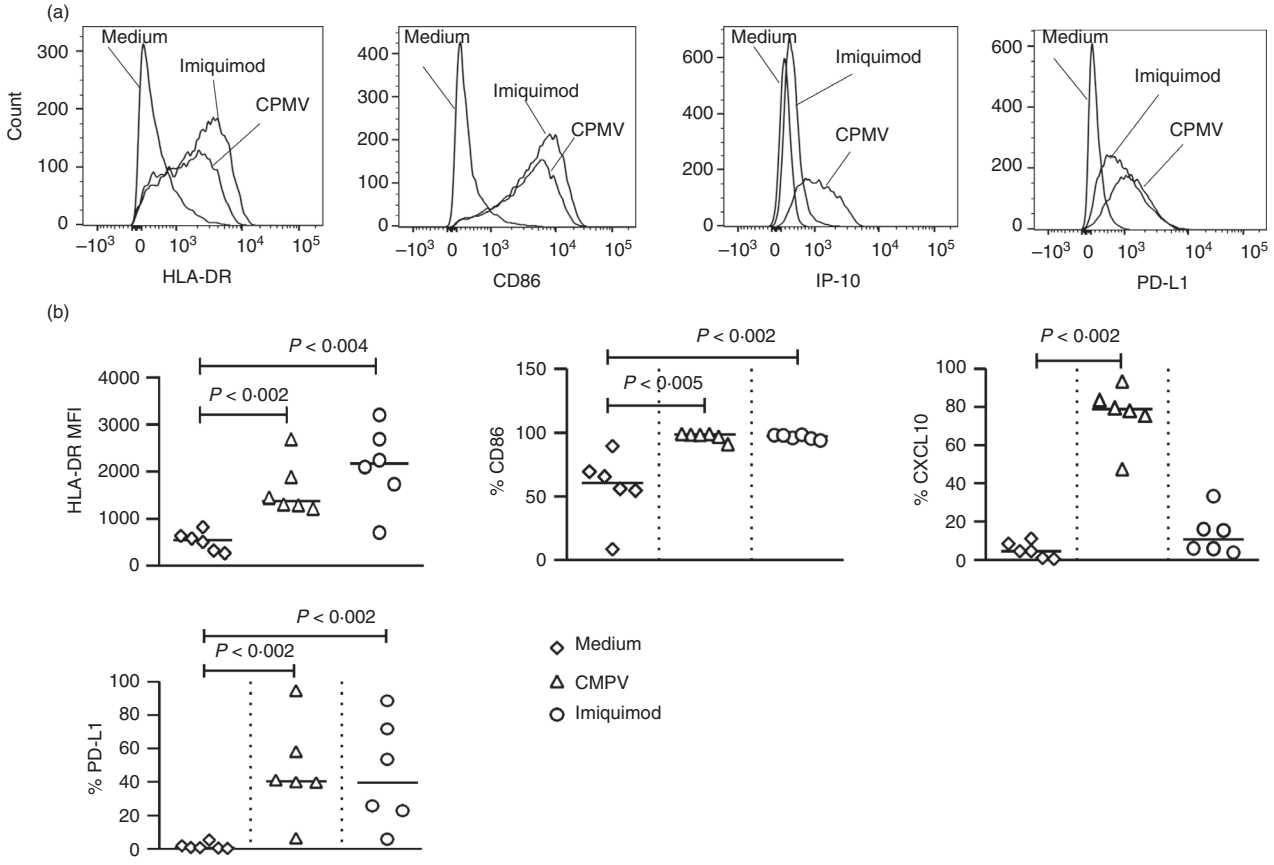


**Figure 2.** Activation of purified monocytes by cowpea mosaic virus (CPMV). Purified monocytes were treated overnight with CPMV (6 μg/ml), Imiquimod (1 μg/ml) or incubated in medium alone. (a) The cells were stained for expression of CD86, PD-L1, HLA-DR, IL-15R or CXCL10 (*x*-axis), and analysed by flow cytometry. Dead cells, debris and doublets were removed from the analysis. (b) Summary data using cells from six different donors. Samples were evaluated using Mann–Whitney *U*-test. *P*-value < 0·007 by Kruskal–Wallis test.

## Discussion

Novel therapeutics that induce anti-tumour immunity such as immune checkpoint inhibitors, chimeric antigen receptor cell therapies and tumour-associated antigen cancer vaccines have shown considerable progress,[21–24] but the development of immunotherapy for cancer is in an early stage and more research and alternative approaches are needed. The potential of virus-based materials for medical applications has been recognized, such as mammalian virus-based nanoparticles for gene therapy and oncolytic virotherapy, which are currently in clinical trials.[25] Despite numerous novel virus-based materials under investigation, plant viruses may have an advantage because they are considered safer in humans than mammalian viruses.[26] Plant viruses do not replicate in or infect mammals, can be administered at doses of up to 100 mg ($10^{16}$ particles) per kg body weight without clinical toxicity,[27,28] and show both biocompatibility and biodegradability.[28–30] Plant viruses and VLPs thereof have been engineered as epitope display platforms and applied in the context of cancer vaccines; for example, the potato virus X (PVX) platform displaying idiotypic (Id), a weak tumour antigen from B-cell lymphoma, successfully protected mice against lymphoma challenge through production of Id-specific antibodies. In addition to its function as epitope display platform, PVX acts as adjuvant and stimulates immune responses through TLR-7 signalling.[31] In alignment with these observations and recent evidence suggests that PapMV mediates activation of human cells *in vitro* and, similar to our findings with CPMV, requires TLR7/8 signalling mechanisms.[32]

We have reported that CPMV nanoparticles can modulate the local microenvironment to relieve immunosuppression and potentiate anti-tumour immunity. The response induced by CPMV can lead to systemic, immune-mediated anti-tumour responses against unrecognized metastases.[33,34] A comparison study between CPMV and another plant virus, tobacco mosaic virus (TMV), indicated that CPMV versus TMV modulates the tumour microenvironment in distinct ways.[34] Interestingly, nucleic acid-free VLPs of CPMV also have demonstrated anti-tumour effects in murine models and canine patients.[7,33]

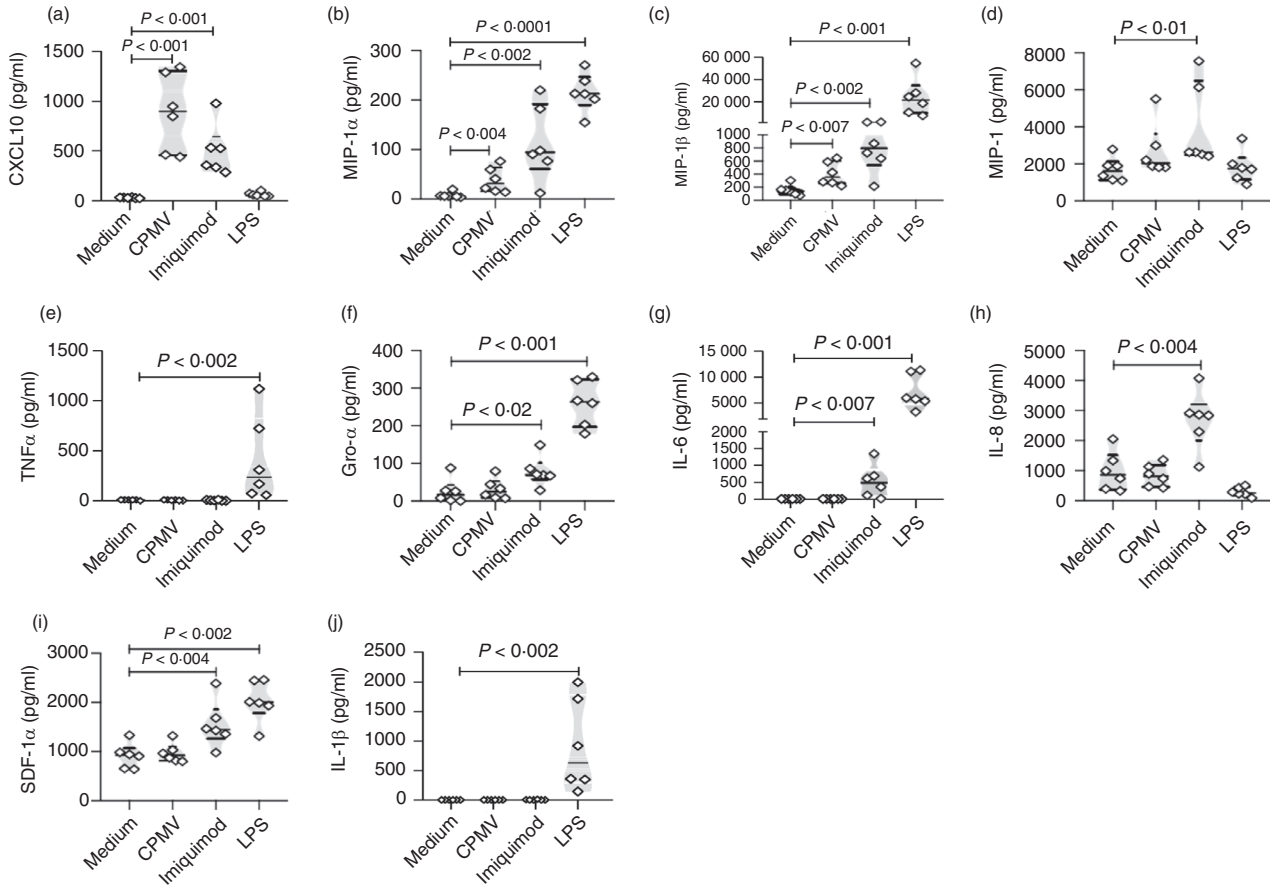


**Figure 3.** Purified monocytes exposed to cowpea mosaic virus (CPMV) produce CXCL10, MIP-1α and MIP-1β *in vitro*. (a–j) Purified monocytes from six different donors were cultured overnight with CPMV (6 μg/ml), Imiquimod (1 μg/ml), lipopolysaccharide (LPS; 50 ng/ml) or incubated in medium alone. Supernatants were collected, stored frozen and assessed in batch with luminex technology to determine cytokine/chemokine concentrations. Summary data showing experiment (symbols) and medium. Samples were evaluated using Mann–Whitney *U*-test. *P*-value < 0·04 by Kruskal–Wallis test.

In this study, we investigated the capacity of CPMV particles to trigger activation of human immune cells, particularly professional APCs. We found that treatment of cells with CPMV induced activation of monocytes, DCs (pDC and mDC) and monocyte-derived macrophages. Most of our data centre on monocyte responses to CPMV. In this context, it is important to recognize that in addition to serving as precursors to tumour-associated macrophages and DCs, monocytes that infiltrate tumours can mediate important effects in this microenvironment, including contributions to tumour development, survival (angiogenesis, immunosuppression) and metastasis.[35–44] In part, this may be due to the capacity of primary monocytes to respond to hypoxic microenvironments in a manner similar to macrophages.[45]

A key objective of this study was to gain insight into the molecular mechanisms underlying CPMV activity. To understand downstream signalling pathways responsible for CPMV activation of monocytes, we investigated the importance of Syk signalling in CPMV-activated monocytes. We found that inhibition of Syk markedly impaired the capacity of CPMV to mediate activation of monocytes. Syk is a tyrosine kinase required in many immune cell signalling pathways. Syk signalling can contribute to cellular activation and cytokine induction,[46–49] and Syk has been implicated in cellular activation mediated by several TLRs such as TLR4, TLR5, TLR7 and TLR9. Syk is thought to contribute to TLR/MYD88 interactions downstream of TLR ligation.[50] Notably, Syk is also implicated in other activation pathways such as C-type Lectin receptor signalling.[48] In preliminary experiments, we were unable to block the activation of monocytes by CPMV using anti-hDectin-1-IgG, which blocks signalling by C-type Lectin receptors (not shown). Given this observation and our other findings, we favour a model whereby the importance of Syk in CPMV activity is linked to endosomal TLR signalling.

TLR7 and TLR8 are endosomal TLRs that have been implicated in the sensing of single-stranded ribonucleic acid,[51] and our data implicate these TLRs as contributors


 

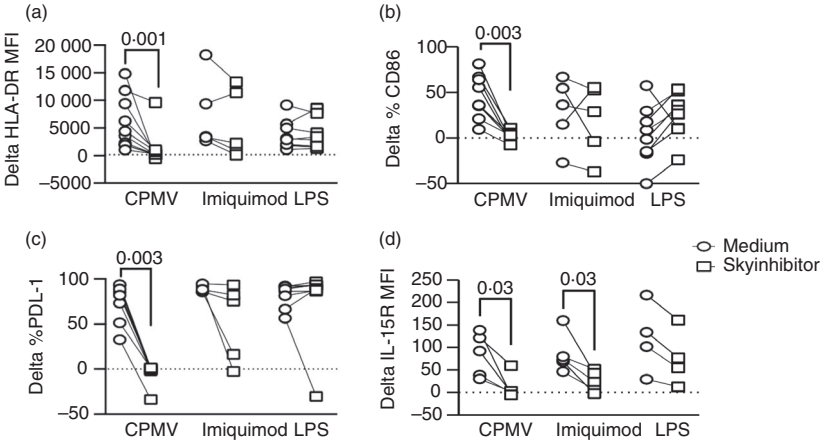


**Figure 4.** Inhibition of spleen tyrosine kinase (Syk) impairs monocyte activation by cowpea mosaic virus (CPMV). Peripheral blood mononuclear cells (PBMCs) were pre-incubated +/− Syk inhibitor (2 μM) for 2 hr. After pre-incubation, cells were stimulated overnight with CPMV (6 μg/ml), Imiquimod (1 μg/ml), lipopolysaccharide (LPS; 50 ng/ml) or incubated in medium alone. Cells were harvested and stained for flow cytometric analysis. Data are derived from analysis of CD14+ cells. The change in MFI (a, d) or change in percent positive PD-L1 or CD86 cells above (or below) background are shown (b, c). The average background MFIs for HLA-DR and IL-15R were 2026 and 94 fluorescent units, respectively, and the mean percent positive PD-L1 or CD86 cells were 7% and 47%, respectively, for cells incubated in resting conditions (medium alone). Statistical analysis was performed with Wilcoxon matched-pairs signed-ranks test.

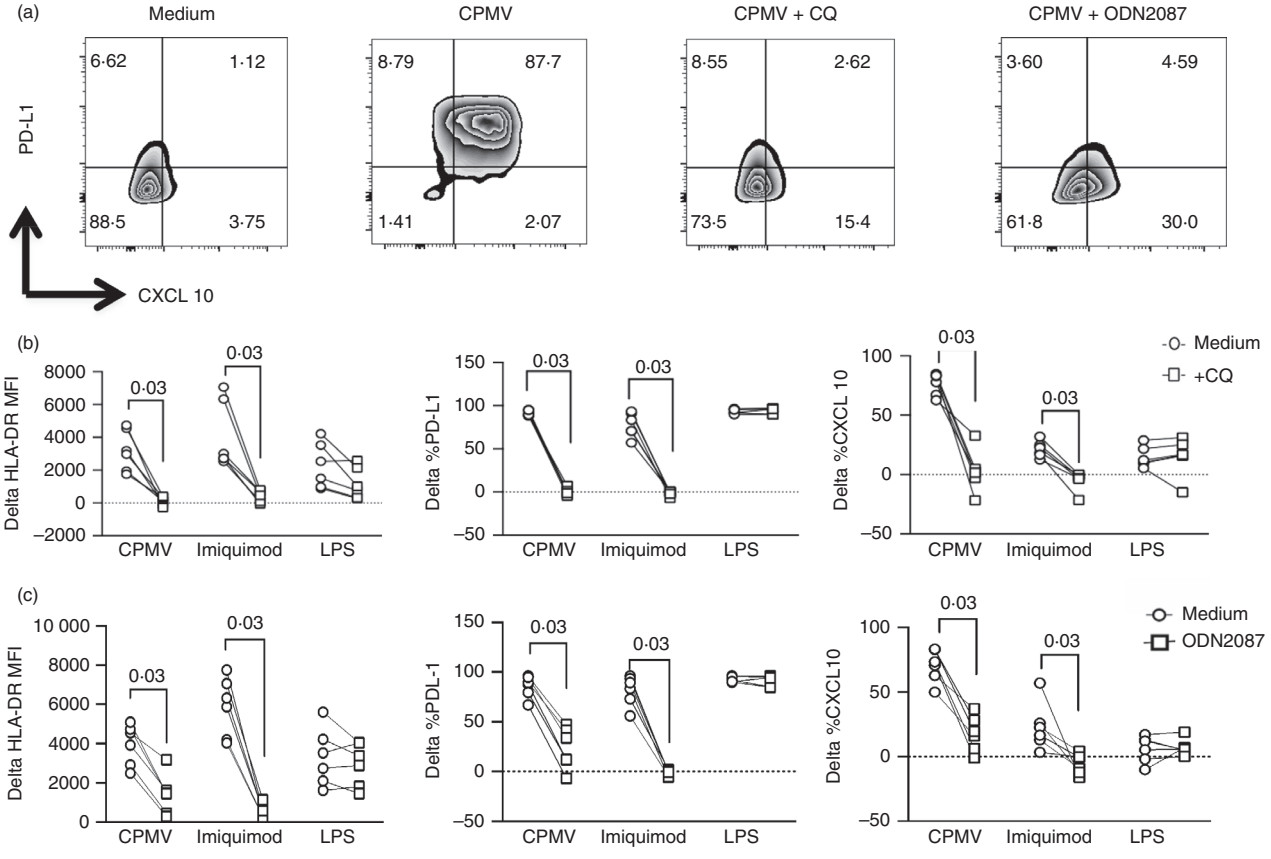


**Figure 5.** Inhibition of endosomal acidification, TLR7/8 or TLR7/8/9 impairs monocyte activation by cowpea mosaic virus (CPMV). Peripheral blood mononuclear cells (PBMCs) were pre-incubated +/− Chloroquine diphosphate (CQ; 10 μM) or ODN 2087 (4–8 μM) for 1 hr. Following pre-incubation, cells were stimulated with CPMV (6 μg/ml), Imiquimod (1 μg/ml), lipopolysaccharide (LPS; 50 ng/ml) or incubated in medium alone overnight. The cells were then stained with various monocyte activation markers, and analysed by flow cytometry. (a) A contour plot for percentage of PD-L1 and CXCL10 expression after stimulation by CPMV, Imiquimod, LPS or medium alone. (b) Summary data for CQ-pretreated PBMCs. (c) Summary data for ODN-pretreated PBMCs, ($n$ = 6) donors. Statistical analyses were performed with Wilcoxon matched-pairs signed-ranks tests.

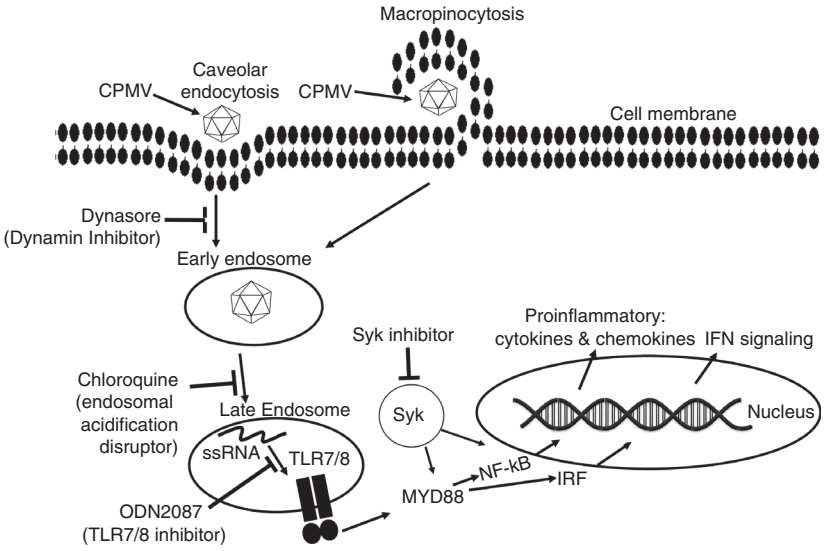


**Figure 6.** Overview of monocyte activation induced by cowpea mosaic virus (CPMV). CPMV mediates monocyte activation via endocytosis (partially blocked by dynasore), endosomal maturation (disrupted by Chloroquine), TLR7/8 (inhibited by ODN2087) and spleen tyrosine kinase (Syk) activation (blocked by Syk inhibitor).

to CPMV activity. CPMV activity was partially blocked by an endocytosis inhibitor (dynasore) that has been demonstrated to partially interfere with CPMV internalization via caveolar-dependent endocytosis.[11,52] CPMV activity was also blocked by an endosomal acidification inhibitor (Chloroquine) and by a TLR7/8 antagonist.[53,54] Moreover, CPMV inhibition by Syk inhibitor resembles the inhibition of a canonical TLR7 agonist, Imiquimod. Overall, these data are consistent with an important role for endocytic TLR signalling in CPMV-mediated activation of primary human cells (summarized in Fig. 6).

Although we have implicated the involvement of TLR7/8 in the activation of human cells by CPMV, we also find evidence that CPMV and a canonical TLR7 agonist, Imiquimod, may have different effects on human monocytes. In particular, we found that CPMV was more efficient at inducing CXCL10 expression in human monocytes than Imiquimod, despite similar induction of other activation indices. Our multiplex analysis for cytokines and chemokines confirmed the robust release of CXCL10 from monocyte following CPMV stimulation. CXCL10 has been shown to be induced in response to type I and II IFNs, and can act as a chemoattractant to CXCR3-expressing immune cells such as monocytes, DCs, NK cells and T-cells.[55–61] Notably, CXCL10 has been found to play a key role in directing T-cell infiltration into solid tumours, making it possible that induction of this chemokine by CPMV in tumour-associated monocytes or macrophages could contribute to *in vivo* antitumour activity of CPMV.[62,63] In contrast to the effects on CXCL10 release, cytokines/chemokines induced by NF-κB-dependent signalling (IL-6, IL-8, IL-1β or TNF-α) were not induced by CPMV. Imiquimod, however, caused the induction of IL-6 and IL-8 in monocytes.[64] This observation raises the possibility that CPMV activates endosomal TLRs in a manner that skews responses towards IRF-signalling, rather than NF-κB-signalling. Perhaps, this might be a consequence of different interactions between TLR7/8 and chemical or viral RNA ligands.[65] Alternatively, CPMV may mediate some of its activity via other receptors that are distinct from TLR7/8. Further studies will be required to discern these possibilities and the molecular mechanisms involved.

## Author contributions

MMA performed experiments, designed studies and wrote the manuscript. SFS designed studies. FAV produced, purified and characterized CPMV particles. SNF and NFS reviewed data and provided input for interpretation of results. All co-authors reviewed the manuscript, providing input for revisions.

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## Disclosure

The authors declare no conflict of interest.

## References

1 Porta C, Spall VE, Lin T, Johnson JE, Lomonossoff GP. The development of cowpea mosaic virus as a potential source of novel vaccines. *Intervirology* 1996; **39**:79–84.

2 Wen AM, Le N, Zhou X, Steinmetz NF, Popkin DL. Tropism of CPMV to professional antigen presenting cells enables a platform to eliminate chronic infections. *ACS Biomater Sci Eng* 2015; **1**:1050–4.
3 Chatterji A, Ochoa W, Shamieh L, Salakian SP, Wong SM, Clinton G *et al.* Chemical conjugation of heterologous proteins on the surface of Cowpea mosaic virus. *Bioconjug Chem* 2004; **15**:807–13.
4 Lewis JD, Destito G, Zijlstra A, Gonzalez MJ, Quigley JP, Manchester M *et al.* Viral nanoparticles as tools for intravital vascular imaging. *Nat Med* 2006; **12**:354–60.
5 Wang C, Fiering SN, Steinmetz NF. Cowpea mosaic virus promotes anti-tumor activity and immune memory in a mouse ovarian tumor model. *Adv Therap* 2019; **2**(5):1 900 003.
6 Kerstetter-Fogle A, Shukla S, Wang C, Beiss V, Harris PL, Sloan AE *et al.* Plant virus-like particle in situ vaccine for intracranial glioma immunotherapy. *Cancers* 2019; **11**:515.
7 Hoopes PJ, Wagner RJ, Duval K, Kang K, Gladstone DJ, Moodie KL *et al.* Treatment of canine oral melanoma with nanotechnology-based immunotherapy and radiation. *Mol Pharm* 2018; **15**:3717–22.
8 Lebel ME, Chartrand K, Tarrab E, Savard P, Leclerc D, Lamarre A. Potentiating cancer immunotherapy using papaya mosaic virus-derived nanoparticles. *Nano Lett* 2016; **16**:1826–32.
9 Lomonossoff G, Johnson J. The synthesis and structure of comovirus capsids. *Prog Biophys Mol Biol* 1991; **55**:107–37.
10 Saunders K, Sainsbury F, Lomonossoff GP. Efficient generation of cowpea mosaic virus empty virus-like particles by the proteolytic processing of precursors in insect cells and plants. *Virology* 2009; **393**:329–37.
11 Plummer EM, Manchester M. Endocytic uptake pathways utilized by CPMV nanoparticles. *Mol Pharm* 2013; **10**:26–32.
12 Agrawal A, Manchester M. Differential uptake of chemically modified cowpea mosaic virus nanoparticles in macrophage subpopulations present in inflammatory and tumor microenvironments. *Biomacromol* 2012; **13**:3320–6.
13 Leong HS, Steinmetz NF, Ablack A, Destito G, Zijlstra A, Stuhlmann H *et al.* Intravital imaging of embryonic and tumor neovasculature using viral nanoparticles. *Nat Protoc* 2010; **5**:1406.
14 Boyum A. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand J Clin Lab Invest Suppl* 1968; **97**:77–89.
15 Lenert PS. Classification, mechanisms of action, and therapeutic applications of inhibitory oligonucleotides for Toll-like receptors (TLR) 7 and 9. *Mediators Inflamm* 2010; **2010**:986 596.
16 Lu X, Masic A, Liu Q, Zhou Y. Regulation of influenza A virus induced CXCL-10 gene expression requires PI3K/Akt pathway and IRF3 transcription factor. *Mol Immunol* 2011; **48**:1417–23.
17 Buttmann M, Berberich-Siebelt F, Serfling E, Rieckmann P. Interferon-beta is a potent inducer of interferon regulatory factor-1/2-dependent IP-10/CXCL10 expression in primary human endothelial cells. *J Vascu Res* 2007; **44**:51–60.
18 Pugazhenthi S, Zhang Y, Bouchard R, Mahaffey G. Induction of an inflammatory loop by interleukin-1beta and tumor necrosis factor-alpha involves NF-kB and STAT-1 in differentiated human neuroprogenitor cells. *PLoS ONE* 2013; **8**:e69585.
19 Harris DP, Bandyopadhyay S, Maxwell TJ, Willard B, DiCorleto PE. Tumor necrosis factor (TNF)-alpha induction of CXCL10 in endothelial cells requires protein arginine methyltransferase 5 (PRMT5)-mediated nuclear factor (NF)-kappaB p65 methylation. *J Biol Chem* 2014; **289**:15 328–39.
20 Aouar B, Kovarova D, Letard S, Font-Haro A, Florentin J, Weber J *et al.* Dual role of the tyrosine kinase Syk in regulation of toll-like receptor signaling in plasmacytoid dendritic cells. *PLoS ONE* 2016; **11**:e0156063.
21 Goel G, Sun W. Cancer immunotherapy in clinical practice – the past, present, and future. *Chin J Cancer* 2014; **33**:445–57.
22 Ma C, Fan R. Cancer immunotherapy and next-generation clinical immune assessment. *Front Oncol* 2014; **4**:265.
23 Sheng W-Y, Huang L. Cancer immunotherapy and nanomedicine. *Pharm Res* 2011; **28**:200–14.
24 Shore ND. Advances in the understanding of cancer immunotherapy. *BJU Int* 2015; **116**:321–9.
25 Liu TC, Galanis E, Kirn D. Clinical trial results with oncolytic virotherapy: a century of promise, a decade of progress. *Nat Clin Pract Oncol* 2007; **4**:101–17.
26 Manchester M, Singh P. Virus-based nanoparticles (VNPs): platform technologies for diagnostic imaging. *Adv Drug Deliv Rev* 2006; **58**:1505–22.
27 Kaiser CR, Flenniken ML, Gillitzer E, Harmsen AL, Harmsen AG, Jutila MA *et al.* Biodistribution studies of protein cage nanoparticles demonstrate broad tissue distribution and rapid clearance *in vivo*. *Int J Nanomedicine* 2007; **2**:715–33.
28 Singh P, Prasuhn D, Yeh RM, Destito G, Rae CS, Osborn K *et al.* Bio-distribution, toxicity and pathology of cowpea mosaic virus nanoparticles *in vivo*. *J Control Release* 2007; **120**:41–50.
29 Bruckman MA, Randolph LN, VanMeter A, Hern S, Shoffstall AJ, Taurog RE *et al.* Biodistribution, pharmacokinetics, and blood compatibility of native and PEGylated tobacco mosaic virus nano-rods and -spheres in mice. *Virology* 2014; **449**:163–73.
30 Lee KL, Shukla S, Wu M, Ayat NR, El Sanadi CE, Wen AM *et al.* Stealth filaments: polymer chain length and conformation affect the *in vivo* fate of PEGylated potato virus X. *Acta Biomater* 2015; **19**:166–79.
31 Jobsri J, Allen A, Rajagopal D, Shipton M, Kanyuka K, Lomonossoff GP *et al.* Plant virus particles carrying tumour antigen activate TLR7 and Induce high levels of protective antibody. *PLoS ONE* 2015; **10**:e0118096.
32 Carignan D, Herblot S, Laliberté-Gagné M-È, Bolduc M, Duval M, Savard P *et al.* Activation of innate immunity in primary human cells using a plant virus derived nanoparticle TLR7/8 agonist. *Nanomedicine* 2018; **14**:2317–27.
33 Lizotte PH, Wen AM, Sheen MR, Fields J, Rojanasopondist P, Steinmetz NF *et al.* In situ vaccination with cowpea mosaic virus nanoparticles suppresses metastatic cancer. *Nat Nanotechnol* 2016; **11**:295–303.
34 Murray AA, Wang C, Fiering S, Steinmetz NF. In situ vaccination with cowpea vs tobacco mosaic virus against melanoma. *Mol Pharm* 2018; **15**:3700–16.
35 De Palma M, Venneri MA, Galli R, Sergi LS, Politi LS, Sampaolesi M *et al.* Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell* 2005; **8**:211–26.
36 Venneri MA, De Palma M, Ponzoni M, Pucci F, Scielzo C, Zonari E *et al.* Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. *Blood* 2007; **109**:5276–85.
37 Bron S, Henry L, Faes-Van'T Hull E, Turrini R, Vanhecke D, Guex N *et al.* TIE-2-expressing monocytes are lymphangiogenic and associate specifically with lymphatics of human breast cancer. *Oncoimmunology* 2016; **5**:e1073882.
38 Ibberson M, Bron S, Guex N, Faes-van't Hull E, Ifticene-Treboux A, Henry L *et al.* TIE-2 and VEGFR kinase activities drive immunosuppressive function of TIE-2–expressing monocytes in human breast tumors. *Clin Cancer Res* 2013; **19**:3439–49.
39 Jung K, Heishi T, Khan OF, Kowalski PS, Incio J, Rahbari NN *et al.* Ly6C lo monocytes drive immunosuppression and confer resistance to anti-VEGFR2 cancer therapy. *J Clin Investig* 2017; **127**:3039–51.
40 Feng X, Szulzewsky F, Yerevanian A, Chen Z, Heinzmann D, Rasmussen RD *et al.* Loss of CX3CR1 increases accumulation of inflammatory monocytes and promotes gliomagenesis. *Oncotarget* 2015; **6**:15 077.
41 Lu X, Kang Y. Chemokine (CC motif) ligand 2 engages CCR2+ stromal cells of monocytic origin to promote breast cancer metastasis to lung and bone. *J Biol Chem* 2009; **284**:29 087–96.
42 Qian B-Z, Li J, Zhang H, Kitamura T, Zhang J, Campion LR *et al.* CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature* 2011; **475**:222.
43 Sanford DE, Belt BA, Panni RZ, Mayer A, Deshpande AD, Carpenter D *et al.* Inflammatory monocyte mobilization decreases patient survival in pancreatic cancer: a role for targeting the CCL2/CCR2 axis. *Clin Cancer Res* 2013; **19**:3404–15.
44 Porrello A, Leslie PL, Harrison EB, Gorentla BK, Kattula S, Ghosh SK *et al.* Factor XIIIA—expressing inflammatory monocytes promote lung squamous cancer through fibrin cross-linking. *Nat Commun* 2018; **9**:1988.
45 Bosco MC, Puppo M, Blengio F, Fraone T, Cappello P, Giovarelli M *et al.* Monocytes and dendritic cells in a hypoxic environment: spotlights on chemotaxis and migration. *Immunobiology* 2008; **213**:733–49.
46 Dennehy KM, Ferwerda G, Faro-Trindade I, Pyż E, Willment JA, Taylor PR *et al.* Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors. *Eur J Immunol* 2008; **38**:500–6.
47 Rogers NC, Slack EC, Edwards AD, Nolte MA, Schulz O, Schweighoffer E *et al.* Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity* 2005; **22**:507–17.
48 Ostrop J, Lang R. Contact, collaboration, and conflict: signal integration of Syk-Coupled C-Type lectin receptors. *J Immunol* 1950; **2017**:1403–14.
49 Chaudhary A, Fresquez TM, Naranjo MJ. Tyrosine kinase Syk associates with toll-like receptor 4 and regulates signaling in human monocytic cells. *Immunol Cell Biol* 2007; **85**:249–56.
50 Lin YC, Huang DY, Chu CL, Lin YL, Lin WW. The tyrosine kinase Syk differentially regulates Toll-like receptor signaling downstream of the adaptor molecules TRAF6 and TRAF3. *Sci Signal* 2013; **6**:ra71.
51 Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol* 2005; **17**:1–14.
52 Macia E, Ehrlich M, Massol R, Boucrot E, Brunner C, Kirchhausen T. Dynasore, a cell-permeable inhibitor of dynamin. *Dev Cell* 2006; **10**:839–50.
53 Al-Bari MAA. Targeting endosomal acidification by chloroquine analogs as a promising strategy for the treatment of emerging viral diseases. *Pharm Res Perspect* 2017; **5**:e00293.
54 Lakadamyali M, Rust MJ, Babcock HP, Zhuang X. Visualizing infection of individual influenza viruses. *Proc Natl Acad Sci USA* 2003; **100**:9280–5.

55 Santodonato L, D'Agostino G, Nisini R, Mariotti S, Monque DM, Spada M *et al*. Monocyte-derived dendritic cells generated after a short-term culture with IFN-α and granulocyte-macrophage colony-stimulating factor stimulate a potent Epstein-Barr virus-specific CD8+ T cell response. *J Immunol* 2003; **170**:5195–202.

56 Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S *et al*. The nature of the principal type 1 interferon-producing cells in human blood. *Science* 1999; **284**:1835–7.

57 Sun S, Zhang X, Tough DF, Sprent J. Type I interferon-mediated stimulation of T cells by CpG DNA. *J Exp Med* 1998; **188**:2335–42.

58 Hilkens CM, Schlaak JF, Kerr IM. Differential responses to IFN-α subtypes in human T cells and dendritic cells. *J Immunol* 2003; **171**:5255–63.

59 Luster AD, Ravetch JV. Biochemical characterization of a gamma interferon-inducible cytokine (IP-10). *J Exp Med* 1987; **166**:1084–97.

60 Wendel M, Galani IE, Suri-Payer E, Cerwenka A. Natural killer cell accumulation in tumors is dependent on IFN-gamma and CXCR3 ligands. *Cancer Res* 2008; **68**:8437–45.

61 Costa C, Traves SL, Tudhope SJ, Fenwick PS, Belchamber KB, Russell RE *et al*. Enhanced monocyte migration to CXCR3 and CCR5 chemokines in COPD. *Eur Respir J* 2016; **47**:1093–102.

62 Mikucki M, Fisher D, Matsuzaki J, Skitzki J, Gaulin N, Muhitch J *et al*. Non-redundant requirement for CXCR3 signalling during tumoricidal T-cell trafficking across tumour vascular checkpoints. *Nat Commun* 2015; **6**:7458.

63 Musha H, Ohtani H, Mizoi T, Kinouchi M, Nakayama T, Shiiba K *et al*. Selective infiltration of CCR5+ CXCR3+ T lymphocytes in human colorectal carcinoma. *Int J Cancer* 2005; **116**:949–56.

64 Liu T, Zhang L, Joo D, Sun SC. NF-kappaB signaling in inflammation. *Signal Transduct Target Ther*. 2017; **2**:e17023.

65 Zhang Z, Ohto U, Shibata T, Krayukhina E, Taoka M, Yamauchi Y *et al*. Structural analysis reveals that toll-like receptor 7 is a dual receptor for guanosine and single-stranded RNA. *Immunity* 2016; **45**:737–48.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Characterization of CPMV particles.

**Figure S2.** Activation of DCs and macrophages by CPMV.

**Figure S3.** Dynasore partially blocked monocyte activation by CPMV.



 